Chromatin fragmentation associated with apoptotic changes in tobacco cells exposed to cold stress

Blažena Koukalová*, Aleš Kovařík, Jiří Fajkus, Jiří Široký

Institute of Biophysics, Academy of Sciences of the Czech Republic, Královopolská 135, CZ-612 65 Brno, Czech Republic

Received 29 April 1997; revised version received 22 July 1997

Abstract Programmed cell death (PCD) may be triggered by a variety of environmental stimuli. In this report we show that low temperature treatment of tobacco BY-2 cells results in specific chromatin changes. The early stage was characterised by chromatin condensation associated with specific endonucleolytic cleavage of the genome into fragments of 50–100 kbp in size. Later, after 2 weeks of the cold treatment, a ladder of nucleosomal units (178 bp) and their multiples occurred. Chromatin changes were accompanied by a general decrease in cell viability. However, the cell culture retained about 11% of living cells even after prolonged incubation in the cold suggesting the presence of a cold-resistant population of cells. The results support the view that PCD was activated by the cold stress. We suggest that cold-stressed tobacco BY-2 culture might be a useful system for investigation of PCD in plant cells.

Key words: Programmed cell death; Cold stress; Chromatin fragmentation; Nucleosomal ladder; Tobacco

1. Introduction

Programmed cell death (PCD), known as apoptosis, seems to be an ubiquitous mechanism used by multicellular organisms for developmental reasons, homeostasis and as a response to adverse situations. Currently, PCD has been described in animal cells in great detail [1] at morphological, biochemical and genetic levels. Morphological alterations exhibited by cells undergoing PCD include cell shrinkage, membrane blebbing, chromatin condensation and fragmentation. PCD in plants is less well understood. However, in recent years evidence has accumulated suggesting that plant organisms activate the cell death program in several distinct situations: (i) during certain developmental transitions, (ii) as a response to some pathogens, such as hypersensitive response and (iii) as a response to an adverse environment ([2–4], and references therein).

Here we report on chromatin changes induced by cold stress in tobacco BY-2 cells. The observed specific chromatin fragmentation coupled with apoptotic changes of the cell nucleus and cytoplasm suggest that PCD might be activated in tobacco cells in response to cold stress.

*Corresponding author. Fax: (42) (5) 41211293.
E-mail: kovarik@ibp.cz

Abbreviations: MNase, micrococcal nuclease; PCD, programmed cell death; PFGE, pulsed field gel electrophoresis

2. Materials and methods

2.1. Plant material and culture conditions

The tobacco bright yellow (BY-2) cell line [5] was propagated in liquid MS medium [6] supplemented with sucrose (3 g/l), thiamine (1 mg/l), KH_2PO_4 (200 mg/l), myoinositol (100 mg/l) and 2,4-dichlorophenoxyacetic acid (0.2 mg/l). Suspension cultures were grown in Erlenmeyer flasks under constant shaking (130 rpm) at 27°C.

Cold stress was induced by transfer of suspension culture (grown to a density of about 10^6 cells/ml) to a cold room (5–6°C). A parallel culture was grown without subcultivation at 27°C. At different time intervals, DNA integrity and cytological changes were monitored.

2.2. Conventional gel electrophoresis and Southern hybridisation

Total DNA was isolated by a slightly modified cetyltrimethylammonium bromide method [7]. BY-2 cells were sedimented and freeze-dried in order to remove excess water prior to grinding in liquid nitrogen. After the isopropanol precipitation step, crude DNA was treated with RNase A and then with proteinase K, phenol-chloroform extracted and precipitated with ethanol. Integrity of DNA was evaluated by electrophoresis in 1.5% agarose gel in the presence of ethidium bromide. Densitometric scanning of gels was performed with a laser densitometer (Molecular Dynamics). Data processing was carried out using ImageQuant software (Molecular Dynamics). Southern hybridisations were performed as described elsewhere [8] using the ^{32}P-labeled HRS60 repetitive probe [9,10] and the internal *Eco*RI fragment of the 25S rDNA gene cloned from the tomato genome [11].

In order to analyse chromatin structure, isolated nuclei were partially digested with micrococcal nuclease (MNase, Boehringer Mannheim). Isolation and digestion of nuclei were performed exactly as described in [12].

2.3. Pulsed field gel electrophoresis

The samples of high-molecular-weight DNA were prepared from protoplasts. About 1 ml of cell culture ($1–5\times10^6$ cells) was pelleted by centrifugation in an Eppendorf centrifuge at $1500\times g$ for 2 min. Cell pellets were resuspended in an excess of a protoplast buffer containing 0.1% pectolyase Y23 and 1% cellulase (both from Seishin Pharmaceutical Co.) in 0.4 M D-mannitol, pH 5.5 and incubated at 26°C for 2 h. The protoplasts were then gently centrifuged and the pellets were mixed with an equal volume of molten 1.5% low gelling temperature agarose in TE (10 mM Tris-HCl, 1 mM EDTA, pH.8.0) buffer and transferred to a mold. After solidification at 4°C, agarose blocks were transferred into a lysis buffer (0.5 M EDTA, pH 9.25, 1% sarcosyl, 0.5 mg/ml proteinase K) and incubated at 37°C for 48 h. The blocks were then washed with TE buffer and used directly, or stored in 0.5 M EDTA at 4°C. Electrophoretic fractionation was carried out in a 1% agarose gel (made from high strength agarose powder, FMC) using CHEF-DR II apparatus (Bio-Rad). The gels were run at 180 V in 45 mM Tris-borate, 1 mM EDTA, pH 8.0 with pulse ramping time from $T_1 = 12$ s to $T_2 = 60$ s at 16°C for 24 h. After electrophoresis, the gel was stained with ethidium bromide (1.0 μg/ml; 30 min) and then destained with distilled water (1 h). The gel was viewed using a UV transilluminator and photographed.

2.4. Cytological methods

The structure of the nuclei was investigated by fluorescence microscopy. Briefly, the cells were fixed in methanol-acetic acid 3:1 (Carnoy's fixative) overnight at −20°C, then transferred onto glass and stained with 1 μg/ml propidium iodide for 5 min. Red fluorescence was visualised using an Olympus epifluorescence microscope. Cell

PII S0014-5793(97)01008-9

viability was identified using fluorescein diacetate which passes across the plasma membrane. Metabolically active protoplasts cleave the compound and release fluorescein [13]. An Olympus epifluorescence microscope was used for the evaluation of the proportion of cells giving yellow fluorescence. Changes in the structure of the cytoplasm were evaluated under phase-contrast light microscopy.

3. Results

3.1. Cold stress-induced fragmentation of nuclear chromatin

An exponentially growing BY-2 cell culture at a density of about 10^6 cells/ml was transferred from the optimal cultivation temperature of 27°C into 5–6°C. Total DNA samples were isolated after 1–5 weeks of cold treatment. The integrity of DNA molecules was monitored by electrophoresis in a 1.5% agarose gel (Fig. 1A). DNA isolated from control cells migrated as an unresolved high molecular weight band of more than 20 kbp in length (lane 1). In contrast, DNA from cells kept at low temperature for 1–5 weeks (lanes 2–6 and 8) showed a significant degree of fragmentation forming ladders of bands. There was apparent progress in DNA fragmentation increasing with the time of cold treatment. After 5 weeks, most of the chromatin was already present as low molecular weight nucleosomal units. Densitometric scanning revealed that the bands were regularly spaced with a periodicity of 178 ± 5 bp, a value close to the size of the tobacco basic chromatin subunit (182 bp); the pattern of tobacco chromatin obtained after 30 min digestion with MNase is given in lane 7. This indicated that cold stress may induce an apoptotic-like endonucleolytic cleavage of nuclear chromatin into oligonucleosomal units. Prolonged cultivation of BY-2 cells at 27°C (2 weeks without transfer to fresh medium, density of 3.6×10^6 cells/ml) caused a smeared DNA pattern without any periodicity (Fig. 1, lane 9). In several studies describing apoptosis in animal cells, characteristic oligonucleosomal fragmentation was shown to be preceded by chromatin cleavage into >50 kbp fragments [14]. Therefore, we examined the integrity of high molecular weight DNA by pulsed field gel electrophoresis (Fig. 1B). DNA from control cells grown at 27°C showed a single compressed band of more than 800 kbp in size. Subsequent degradation upon cold treatment resulted in the appearance of 50–100 kbp fragments. These fragments could be detected even at intervals where no internucleosomal cleavage was visible on conventional electrophoresis. Smeared patterns observed after 4 days of

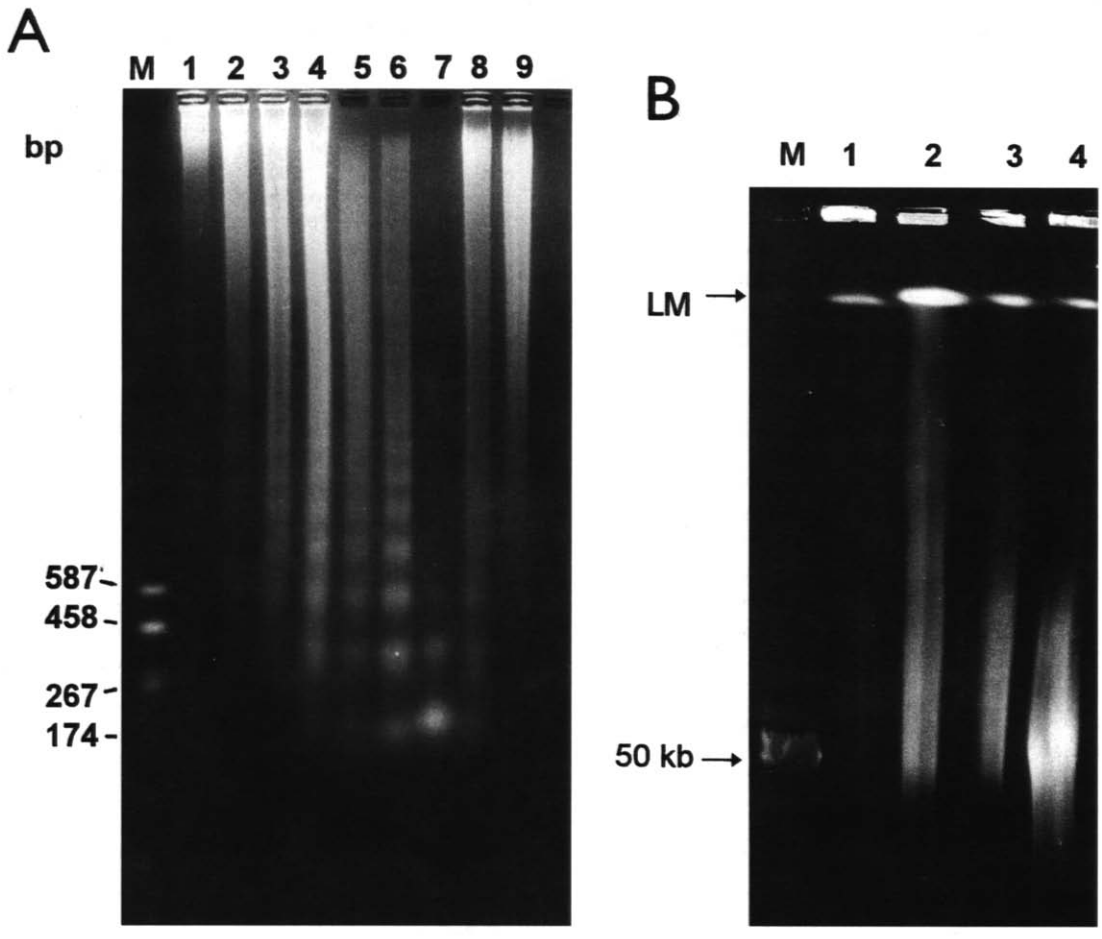

Fig. 1. DNA fragmentation in tobacco BY-2 cells after cold treatment. A: Conventional agarose electrophoresis. Lane 1, control DNA from cells harvested at exponential phase of growth; lanes 2–6, DNAs from cultures exposed to 5–6°C for 1–5 weeks, respectively; lane 7, nucleosomal ladder of tobacco chromatin generated by a 30 min digestion of nuclei with MNase; lanes 8 and 9, DNAs from cultures incubated for 2 weeks at 5–6°C and 27°C, respectively; M, DNA markers. B: Pulsed field gel electrophoresis. High molecular weight DNA embedded in agarose blocks was separated by PFGE under the conditions described in Section 2. Lane 1, control DNA isolated from cells grown at normal cultivation conditions; lanes 2–4, DNA from cultures exposed to 5–6°C for 4, 7, and 14 days, respectively.

cold stress (lane 2) were transient and disappeared after longer incubation intervals.

In order to study DNA fragmentation in defined sequences of the nuclear genome, we hybridised the fragmented DNAs with the HRS60 (representing the subtelomeric repetitive sequence) [9,15,16] and with the ribosomal (25S rDNA) [11] probes. Fig. 2 shows the hybridisation patterns with the HRS60 DNA probe. Hybridisation with the 25S rDNA probe gave the same result (not shown).

3.2. Cytological studies

The above results (Figs. 1 and 2) demonstrated that DNA fragmentation occurred in BY-2 cells exposed to cold stress, suggesting that PCD was induced. Since apoptotic changes, well described in animal cells, are characterised also by destruction of the structural organisation of the nucleus, we studied the morphology of cell nuclei by fluorescence microscopy. BY-2 cells incubated at 5–6°C were fixed and stained with propidium iodide to visualise nuclear chromatin. Fig. 3B shows several characteristic features of nuclei from cold-stressed cells: in comparison with nuclei of control cells (Fig. 3A), chromatin is more condensed and sharply circumscribed, often concentrated at the nuclear membrane or on only one side of the nucleus. These changes occurred after a few days of cold treatment. Fig. 3B shows nuclei of cells cold-treated for 1 week. The DNA pattern of these cells is given in Fig. 1, lane 2.

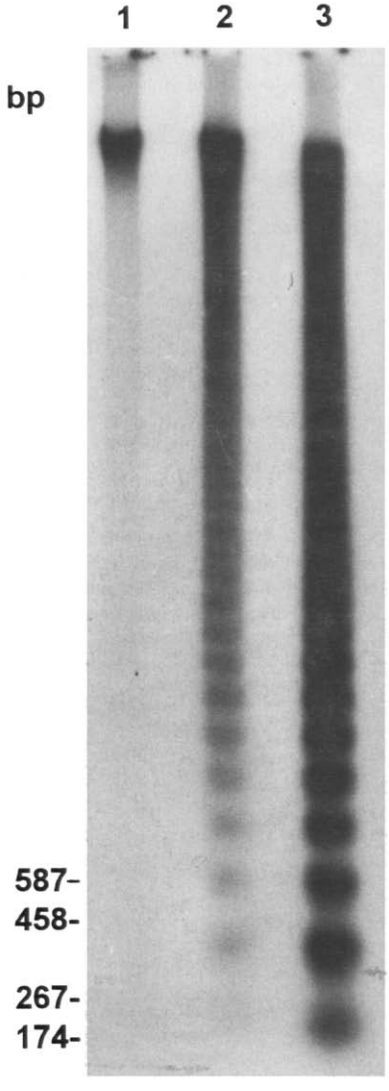

Fig. 2. DNA fragmentation at the HRS60 genomic loci. DNAs shown in Fig. 1, lanes 1, 3 and 6, respectively, were blotted onto a nylon membrane and hybridised to a ^{32}P-labeled HRS60 DNA probe.

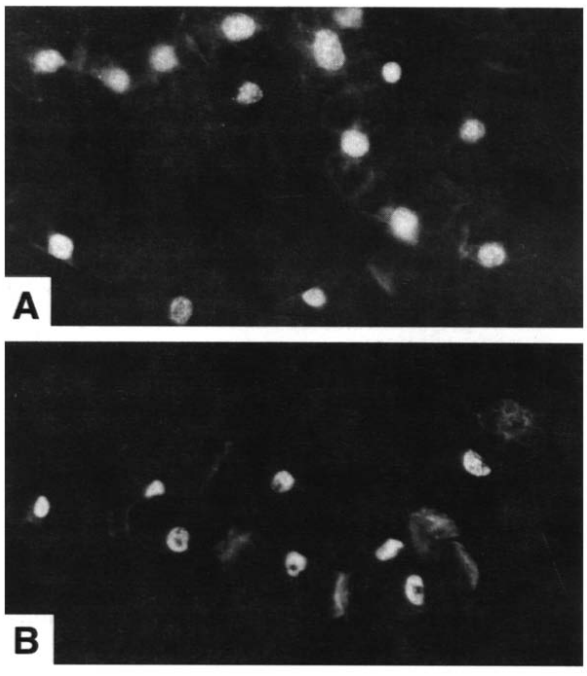

Fig. 3. Fluorescence microscopy of normal and cold-stressed cells of BY-2 cell culture. The cells were fixed with Carnoy's reagent, stained with propidium iodide and photographed under a Olympus epifluorescence microscope. A: Control cells. B: Cells maintained in the cold for 1 week. Bar in A = 50 μm.

Substantial changes also occurred in the cytoplasm. Fluorescein diacetate staining, revealing the metabolic activity of cytoplasm, showed that the proportion of metabolically active cells gradually decreased during the cold treatment. In an early, 4 day interval of the cold treatment, the proportion of active cells fell to about 30%. This decrease continued and reached values ranging from 17 to 11% in time intervals of 2–5 weeks of cold treatment. It should be stressed that even at the time intervals of 4 and 5 weeks of cold treatment, about 11% of cells remained metabolically active. The structure of the cytoplasm, observed under phase-contrast light microscopy, was changed in accordance with the loss of metabolic activity. It became rough and shrinkage of protoplasts occurred. No such changes were visible in metabolically active cells.

4. Discussion

The tobacco BY-2 cell line represents a cell population of high homogeneity thus being convenient for studies of environmental impacts on plant cells. In this report we provide evidence for specific apoptotic changes induced with cold treatment. Ladders of regularly spaced DNA fragments were observed after 2–5 weeks of incubation of the cell culture at 5–6°C. The ladders (spaced at 178 bp) were indistinguishable from those obtained with MNase digestion of nuclear tobacco chromatin suggesting that nucleosomal linker regions were

preferential targets of nuclease cleavage. Two distinct genomic loci, the repetitive HRS60 sequences of the subtelomeric heterochromatin [9,15,16] and 25S rDNA genes [11], were fragmented in the same way as the bulk chromatin suggesting that there was no apparent sequence discrimination in the process of DNA cleavage. PFGE analysis of high molecular weight DNA provided evidence that earlier changes in the integrity of DNA preceded internucleosomal cleavage. The genome digestion into fragments of 50–100 kbp in size was apparent in early intervals of cold treatment (4–7 days) defined by characteristic changes in chromatin condensation, however without any visible DNA 'ladders'. The first nucleosomal ladders emerged after 2 weeks of cold treatment when most DNA was already fragmented into 50–100 kbp fragments. We conclude that internucleosomal cleavage of tobacco chromatin is a late event in response to cold stress and is preceded by genome degradation into 50–100 kbp fragments which may arise from the release of chromatin loops from the nuclear scaffold. This result may provide another parallel with the apoptotic process seen in animal cells where high molecular weight fragmentation is considered an early stage of apoptosis [14]. It should be noted that even after 5 weeks of cold treatment there was a fraction of intact high molecular weight DNA.

Another effect of low temperature concerned the morphology and metabolic activity of cells. Shrinkage of protoplasts was associated with the loss of the cells' ability to cleave fluorescein diacetate. The cell nucleus underwent a number of morphological changes including chromatin condensation, migration of chromatin towards the nuclear membrane and appearance of DNA-free regions in the nucleus. However, a fraction of cells without apoptotic changes was detected even in late stages of cold treatment. This may correspond to the part of cleavage-resistant high molecular weight DNA (Fig. 1A,B). These observations may reflect the presence of a cold stress-resistant population of tobacco cells. Indeed, transfer of cold-stressed cells to a normal growth temperature resulted in a slow culture recovery (not shown).

Plants have to cope with a number of environmental stresses like plant pathogens, elevated or decreased temperature, osmotic stress. Some of these factors have been suggested to activate the cell death program and, recently, the gene ACD2, involved in the cell death program, was identified in *Arabidopsis thaliana* [2]. Apoptotic changes, including DNA fragmentation, were described in tomato cells as a response to host-selective toxins, arachidonic acid, a fungal elicitor of the hypersensitive response from *Phytophthora infestans*, KCN and heat shock [4]. In our experiments, we used an in vitro system, the tobacco BY-2 cell culture. Commonly, in vitro systems offer a possibility to study PCD at the biochemical and molecular levels in a rather homogeneous population of cells. For example, embryogenic carrot cell suspension cultures were conveniently used for PCD studies [17]. A question arises as to whether the chromatin fragmentation and condensation observed in cultured tobacco cells occur also in plants. We believe that a parallel might exist as *Nicotiana tabacum* belongs to cold-sensitive plants. We can conclude that tobacco cells exposed to cold stress undergo specific apoptotic changes, i.e. non-random degradation of nuclear chromatin and characteristic morphological changes of cytoplasm and nuclei, suggesting that the cell death program was activated.

Acknowledgements: This work was supported by the Grant Agency of the Czech Republic (Grants 506/95/0353,521/96/1123 and 521/96/k117). We thank Dr Dennis Francis (University of Cardiff) and Dr. Zdeněk Opatrný for kindly providing the BY-2 cell line, Mrs M. Koudelková and L. Jedličková for technical assistance, Dr M. Bezdik and Dr V. Kleinwächter for critically reading the manuscript and Dr A. Španová and Dr L. Havel for helpful discussions.

References

[1] Vaux, D.L. and Strasser, A. (1996) Proc. Natl. Acad. Sci. USA 93, 2239–2244.
[2] Greenberg, J.T., Guo, A., Klessig, D.F. and Ausubel, F.M. (1994) Cell 77, 551–563.
[3] Ryerson, D.E. and Heat, M.C. (1996) Plant Cell 8, 393–402.
[4] Wang, H., Li, J., Bostock, R.M. and Gilchrist, D.G. (1996) Plant Cell 8, 375–391.
[5] Nagata, T., Okada, K. and Takebe, I. (1982) Plant Cell Rep. 1, 250–252.
[6] Murashige, T. and Skoog, F. (1962) Physiol. Plant. 15, 473–497.
[7] Saghai-Maroof, M.A., Soliman, K.M., Jorgensen, R.A. and Allard, R.W. (1984) Proc. Natl. Acad. Sci. USA 81, 8014–8018.
[8] Sambrook, J., Fritsh, E.F. and Maniatis, T. (1988) Molecular Cloning: A Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[9] Koukalová, B., Reich, J., Matyášek, R., Kuhrová, V. and Bezdìk, M. (1989) Theor. Appl. Genet. 78, 77–80.
[10] Matyášek, R., Koukalová, B. and Reich, J. (1989) Nucleic Acids Res. 17, 4377.
[11] Kiss, T., Kis, M. and Solymosy, F. (1989) Nucleic Acids Res. 17, 796.
[12] Espinás, M.L. and Carballo, M. (1993) Plant Mol.Biol. 21, 847–857.
[13] Power, L.B., Chapman, J.V. and Wilson, D. (1984) Laboratory Manual Plant Tissue Culture, Nottingham.
[14] Oberhammer, F., Wilson, J.W., Dive, C., Morris, I.D., Hickman, J.A., Wakeling, A.E., Walker, P.R. and Sikorska, M. (1993) EMBO J. 9, 3679–3684.
[15] Kenton, A., Parokonny, A.S., Gleba, Y.Y. and Bennet, M.D. (1993) Mol. Gen. Genet. 240, 159–169.
[16] Fajkus, J., Královics, R., Kovařík, A. and Fajkusová, L. (1995) FEBS Lett. 364, 33–35.
[17] McCabe, P.F. and Pennell, R.I. (1996) in: T.G. Cotter and S.J. Martin (Eds.), Techniques in Apoptosis, A User's Guide. Portland Press, London, pp. 302–326.